

08/663618

SOLE INVENTOR



"EXPRESS MAIL" mailing label No.
EM118658816US.

Date of Deposit: June 14, 1996

I hereby certify that this paper (or fee) is being
deposited with the United States Postal Service
"EXPRESS MAIL POST OFFICE TO ADDRESSEE"
service under 37 CFR §1.10 on the date
indicated above and is addressed to:
Assistant Commissioner for Patents, Washington,
D.C. 20231

Li-Hsien Rin-Laures
Li-Hsien Rin-Laures

APPLICATION FOR
UNITED STATES LETTERS PATENT

S P E C I F I C A T I O N

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Patrick W. Gray a citizen of United States,
residing at 2244 38th Place East, Seattle 98112, in the County of King and
State of Washington, have invented a new and useful CHITINASE MATERIALS
AND METHODS, of which the following is a specification.

08663618-082896



\$2,100.00 101 A
08/663618 Bat
Sag

CHITINASE MATERIALS AND METHODS

FIELD OF THE INVENTION

The present invention relates generally to human chitinase enzyme and more specifically to novel purified and isolated polynucleotides encoding human chitinase, to the chitinase products encoded by the polynucleotides, to materials and methods for the recombinant production of chitinase products and to antibody substances specific for the chitinase.

BACKGROUND

Chitin is a linear homopolymer of β -(1,4)-linked N-acetylglucosamine residues. This polysaccharide is second only to cellulose as the most abundant organic substance. The exoskeleton of arthropods is composed of chitin. In addition, fungi and other parasites contain chitin in their outer cell wall, where it serves important structural and protective roles. Disruption of the fungal cell wall and membrane has been a useful therapeutic strategy against fungi and parasites. For example, Amphotericin B and fluconazole exert their anti-fungal activity by affecting membrane steroids. Despite the existence of anti-fungal therapeutics, fungal infections of humans have increasingly become responsible for life-threatening disorders. See, Georgopapadakou *et al.*, *Trends Microbiol.*, 3: 98-104 (1995). The fungal species and parasites responsible for these diseases are mainly *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Coccidioides* and *Pneumocystis*. These pathogens are particularly dangerous in immunocompromised individuals, such as patients with AIDS, patients undergoing chemotherapy, and immunosuppressed organ transplant patients.

Chitin can be degraded by the enzyme chitinase. Chitinase enzymes are found in plants, microorganisms, and animals. Bacterial chitinase helps to provide a carbon source for bacterial growth. Insects produce chitinase to digest their cuticle at each molt. In plants, chitinase is thought to provide a protective role against parasitic fungi. Chitinases have been cloned from numerous bacterial [*e.g.*, *Serratia marcescens*, Jones *et al.*, *EMBO J.*, 5:467-473 (1986)], plant [*e.g.*, tobacco,

08663618-082896

Heitz et al., *Mol. Gen. Genet.*, 245:246-254 (1994)], and insect [*e.g.*, wasp, Krishnan et al., *J. Biol. Chem.*, 269:20971-20976 (1994)] species.

Several proteins with low homology to bacterial, insect, and plant chitinases (less than 40% amino acid identity) have been identified in mammals, such as human cartilage gp-39 (C-gp39) [Hakala et al., *J. Biol. Chem.*, 268:25803-25810 (1993)], human glycoprotein YKL-40 [Johansen et al., *Eur. J. Cancer*, 31A:1437-1442 (1995)], oviduct-specific, estrogen-induced protein from sheep [DeSouza et al., *Endocrinology*, 136:2485-2496 (1995)], cows and humans; and a secretory protein from activated mouse macrophages [Chang et al., Genbank M94584]. However, chitin-degrading activity has not been reported for these proteins. The function of these proteins is not known, but they have been postulated to be involved in tissue remodeling. Hakala et al., *supra*, report that C-gp39 is detectable in synovial and cartilage specimens from rheumatoid arthritis patients, but not from normal humans. Recklies et al., *Arthritis Rheumatism*, 36(9 SUPPL.):S190 (1993) report localization of the C-gp39 protein to a distinct population of cells in the superficial layers of cartilage. Johansen et al., *supra*, report that measurements of YKL-40 serum levels are of value as a potential prognostic marker for the extent of metastatic disease and survival of patients with recurrent breast cancer.

Escott et al., *Infect. Immun.*, 63:4770-4773 (1995) demonstrated chitinase enzymatic activity in human leukocytes and in human serum. Overdijk et al., *Glycobiology*, 4:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema et al., *J. Biol. Chem.*, 270:2198-2202 (February 1995) prepared a human chitotriosidase from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of human chitinase is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same group [Boot et al., *J. Biol. Chem.*, 270(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial

amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product.

In view of the increasing incidence of life-threatening fungal infection in immunocompromised individuals, there exists a need in the art to identify and isolate polynucleotide sequences encoding human chitinases, to develop materials and methods useful for the recombinant production of the enzyme, and to generate reagents for the detection of the chitinase in plasma.

SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotides (*i.e.*, DNA and RNA, both sense and antisense strands) encoding human chitinase or fragments and analogs thereof; methods for the recombinant production of chitinase polypeptides, fragments and analogs thereof; purified and isolated chitinase polypeptide fragments and analogs; antibodies to such polypeptides, fragments and analogs; and pharmaceutical compositions comprising these polypeptides, fragments, analogs, or antibodies.

Specifically provided are: purified, isolated polynucleotides encoding the human chitinase amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; DNAs comprising the protein coding nucleotides of SEQ ID NOS: 1 or 3, particularly nucleotides 65 to 1402 of SEQ ID NO: 1 or nucleotides 90 to 1427 of SEQ ID NO: 3; purified, isolated polynucleotides comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NOS: 14 or 15; purified, isolated polynucleotides encoding human chitinase selected from the group consisting of: (a) a double-stranded DNA comprising the protein coding portions of the sequence set out in SEQ ID NO: 1, (b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a), and (c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b); vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with

such DNAs in a manner allowing the expression in said host cell of human chitinase;
a method for producing human chitinase comprising culturing such host cells in a
nutrient medium and isolating human chitinase from said host cell or said nutrient
medium; purified, isolated polypeptides produced by this method; purified, isolated
5 polypeptides comprising the human chitinase amino acid sequence of SEQ ID NOS: 2
or 4, particularly amino acids 1 to 445 thereof; human chitinase fragments lacking
from 1 to about 72 C-terminal amino acid residues of mature human chitinase,
particularly the human chitinase fragment of SEQ ID NO: 14; the human chitinase
analog of SEQ ID NO: 15; hybridoma cell lines producing a monoclonal antibody that
10 is specifically reactive with one of the above-described polypeptides; and monoclonal
antibodies produced by such hybridomas.

Preferred DNA sequences of the invention include genomic and cDNA
sequences as well as wholly or partially chemically synthesized DNA sequences. The
nucleotide sequence of two human cDNAs encoding presumed allelic variants of
15 human chitinase, and including noncoding 5' and 3' sequences, are set forth in SEQ
ID NO: 1 and SEQ ID NO: 3. These DNA sequences and DNA sequences which
hybridize to the noncoding strand thereof under standard stringent conditions or which
would hybridize but for the redundancy of the genetic code, are contemplated by the
invention. Preferred DNAs of the present invention comprise the human chitinase
20 coding region (corresponding to nucleotides 2 to 1402 of SEQ ID NO: 1 or
nucleotides 27 to 1427 of SEQ ID NO: 3), and the putative coding sequence of the
mature, secreted human chitinase protein without its signal sequence (nucleotides 65
to 1402 of SEQ ID NO: 1, or nucleotides 90 to 1427 of SEQ ID NO: 3).

Exemplary stringent hybridization conditions are as follows:
25 hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1%
SDS. It is understood by those of skill in the art that variation in these conditions
occurs based on the length and GC nucleotide base content of the sequences to be
hybridized. Formulas standard in the art are appropriate for determining exact
hybridization conditions. See Sambrook *et al.*, 9.47-9.51 in *Molecular Cloning*, Cold
30 Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Two amino acid sequences for human chitinase(s) are set forth in SEQ ID NOS: 2 and 4. The sequence of SEQ ID NO: 2 is encoded by the nucleotide sequence of SEQ ID NO: 1, and SEQ ID NO: 4 is encoded by the nucleotide sequence of SEQ ID NO: 3. Preferred polynucleotides of the present invention include, in addition to those polynucleotides described above, polynucleotides that encode amino acids -21 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4, and that differ from the polynucleotides described in the preceding paragraphs only due to the well-known degeneracy of the genetic code. Similarly, since twenty-one amino acids (positions -21 to -1) of SEQ ID NOS: 2 and 4 comprise a signal peptide that is cleaved to yield the mature human chitinase protein, preferred polynucleotides include those encoding polypeptides comprising amino acids 1 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4.

Among the uses for the polynucleotides of the present invention is use as a hybridization probe, to identify and isolate genomic DNA encoding human chitinase; to identify and isolate non-human genes encoding proteins homologous to human chitinase; to identify human and non-human proteins having similarity to human chitinase (including those that may be involved in tissue remodeling); and to identify those cells which express human chitinase and the biological conditions under which this protein is expressed.

In another aspect, the invention includes biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating chitinase polynucleotides, including any of the DNAs described above, are provided. Preferred vectors include expression vectors in which the incorporated chitinase-encoding cDNA is operatively linked to an endogenous or heterologous expression control sequence and a transcription terminator. Such expression vectors may further include polypeptide-encoding DNA sequences operably linked to the chitinase-encoding DNA sequences, which vectors may be expressed to yield a fusion protein comprising the polypeptide of interest.

According to another aspect of the invention, procaryotic or eucaryotic host cells are stably transformed or transfected with DNA sequences of the invention

08653618.002006

in a manner allowing the desired chitinase product to be expressed therein. [Host cells expressing chitinase products can serve a variety of useful purposes. Such cells constitute a valuable source of immunogen for the development of antibody substances specifically immunoreactive with chitinase.] Host cells of the invention are useful in methods for the large scale production of chitinase wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated, *e.g.*, by immunoaffinity purification, from the cells or from the medium in which the cells are grown.

Chitinase products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving procaryotic or eucaryotic host cells of the invention. [Chitinase products of the invention may be full length polypeptides, fragments or analogs thereof.] Chitinase products having part or all of the amino acid sequence set out in SEQ ID NO: 2 or SEQ ID NO: 4 are contemplated. One preferred fragment which lacks the C-terminal seventy-two amino acid residues of the mature protein is set forth in SEQ ID NO: 14. It has been determined that these seventy-two C-terminal residues are not critical to chitinase enzymatic activity. Example 5 illustrates production of this C-terminal fragment; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific activity when compared with full length recombinant human chitinase.

Analogues may comprise chitinase analogs wherein one or more of the specified (*i.e.*, naturally encoded) amino acids is deleted or replaced or wherein one or more nonspecified amino acids are added: (1) without loss of one or more of the enzymatic activities or immunological characteristics specific to chitinase; or (2) with specific disablement of a particular biological activity of chitinase. Example 3 illustrates the production of such an analog (SEQ ID NO: 15), in which the proline at position 370 is substituted with a serine, and in which the C-terminal seventy-two amino acid residues have been deleted. The use of mammalian host cells is also expected to provide for post-translational modifications (*e.g.*, myristolation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation)

as may be needed to confer optimal biological activity on recombinant expression products of the invention.

08663618.082896

5 Proteins or other molecules that bind to chitinase may be used to modulate its activity. Also comprehended by the present invention are antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and the like) and other binding proteins specific for chitinase. Proteins or other molecules (e.g., small molecules) which specifically bind to chitinase can be identified using chitinase isolated from plasma, recombinant chitinase, chitinase variants or cells expressing such products. Binding 10 proteins are useful, in turn, in compositions for immunization as well as for purifying chitinase, and are useful for detection or quantification of chitinase in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for chitinase-specific antibody substances are also contemplated.

15 The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding chitinase and chitinase expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out 20 with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding human allelic variants of chitinase, other structurally related human proteins sharing one or more of the biochemical and/or immunological properties of chitinase, and non-human species proteins homologous to chitinase. The DNA sequence information provided by the 25 present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, e.g., Kapecchi, *Science*, 244: 1288-1292 (1989)], of rodents that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells 30 to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus

that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

08653618.002896

Administration of chitinase preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating disease states caused by chitin-containing parasites such as fungi is contemplated by the invention. Pathogenic fungi cause serious, often fatal disease in immunocompromised hosts. Cancer patients undergoing chemotherapy, immunosuppressed individuals, and HIV-infected individuals are susceptible to mycoses caused by *Candida*, *Aspergillus*, *Pneumocystis carinii*, and other fungi. Amphotericin B and fluconazole are useful therapeutics for fungal infections, but toxicity associated with these drugs causes serious adverse side effects that limit their usefulness. The mortality of systemic candidiasis is greater than 50% despite Amphotericin B treatment. Therefore, a need exists for agents that inhibit fungal growth *in vivo*; and such products may be used as single agents or in combination with currently approved, conventional anti-fungal compounds. Because growing fungi require chitin synthesis for survival, inhibition by recombinant human chitinase may be useful for limiting fungal infections *in vivo*. Animal models for fungal infection are illustrated below in Examples 8 through 14 and have been described in the art.

Specifically contemplated by the invention are chitinase compositions for use in methods for treating a mammal susceptible to or suffering from fungal infections comprising administering chitinase to the mammal in an amount sufficient to supplement endogenous chitinase activity. It is contemplated that the chitinase may be administered with other conventional anti-fungal agents, including amphotericin B and the structurally related compounds nystatin and pimaricin; 5-fluorocytosine; azole derivatives such as fluconazole, ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, itraconazole and tioconazole; allylamines-thiocarbamates, such as tolnaftate, naftifine and terbinafine; griseofulvin; ciclopirox olamine; haloprogin; undecylenic acid; and benzoic acid. [See, *e.g.*, Goodman & Gilman, *The Pharmacological Basis of Therapeutics*, 9th ed., McGraw-Hill, NY (1996).] Chitinase may improve the effectiveness of these conventional

anti-fungal agents, perhaps by rendering the yeast more susceptible to their action, even in situations where the chitinase alone is not effective for inhibiting growth of fungi. By reducing the amount of conventional anti-fungal agent needed to exert the desired therapeutic effect, chitinase may allow the drugs to be used at less toxic levels.

Therapeutic/pharmaceutical compositions contemplated by the invention include chitinase and a physiologically acceptable diluent or carrier and may also include other anti-fungal agents. Dosage amounts indicated would be sufficient to supplement endogenous chitinase activity. For general dosage considerations see *Remington: The Science and Practice of Pharmacy*, 19th ed., Mack Publishing Co., Easton, PA (1995). Dosages will vary between about 1 μ g/kg to 100 mg/kg body weight, and preferably between about 0.1 to about 20 mg chitinase/kg body weight. Therapeutic compositions of the invention may be administered by various routes depending on the infection to be treated, including via subcutaneous, intramuscular, intravenous, intrapulmonary, transdermal, intrathecal, topical, oral, or suppository administration.

The invention also contemplates that the overexpression of chitinase in Gaucher disease or at sites of inflammation (such as in rheumatoid arthritis) may have deleterious effects on the extracellular matrix and, in such disease settings, inhibitors of chitinase activity may provide therapeutic benefit, *e.g.* by reducing remodeling or destruction of the extracellular matrix.

The human chitinase CDNA of the present invention was isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., *Cell*, 85:307-310 (1996)], and macrophage products such as TNF- α are implicated in disease progression. A protein with homology to human chitinase, C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for human chitinase is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extracellular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino et al., *Proc. Nat'l*

Acad. Sci., 93:4548-4553 (1996); Varki, *Proc. Nat'l. Acad. Sci.*, 93:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

Also contemplated by the invention are methods for screening for inhibitors of chitinase activity, which may be useful in the manner described in the preceding paragraph. A method for screening samples to identify agents that inhibit chitinase is reported in, e.g., WO 95/34678 published 21 December 1995.

Further contemplated are methods for measuring endogenous levels of chitinase, e.g., for diagnosing Gaucher's disease. Hollak et al., *J. Clin. Invest.*, 93:1288-1292 (1994), report that plasma chitinase levels are a diagnostic marker for Gaucher's disease. The recombinant proteins of this invention are expected to be more useful than preparations purified from humans, which have associated problems of yield and contamination with other impurities or infectious agents.

DETAILED DESCRIPTION

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of human chitinase cDNA clones from a human macrophage cDNA library. Example 2 addresses the pattern of chitinase gene expression in various human tissues. Example 3 describes the recombinant expression of the human chitinase gene in prokaryotic cells and purification of the resulting enzyme. Example 4 provides a protocol for the recombinant production of human chitinase in yeast. Example 5 describes the recombinant expression of the human chitinase gene in mammalian cells and purification of the resulting protein. Example 6 describes

production of human chitinase polypeptide analogs by peptide synthesis or recombinant production methods. Example 7 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with human chitinase. Example 8 describes an assay for the measurement of chitinase catalytic activity. Example 9 addresses determination of the anti-fungal activity of human chitinase *in vitro*. Example 10 addresses determination of the anti-fungal activity of human chitinase *in vivo* in a mouse model, and Examples 11 through 14 address rabbit models of invasive aspergillosis, disseminated candidiasis, *Candida* ophthalmitis, and *Candida* endocarditis.

Example 1

Isolation of Chitinase cDNA Clones

A cDNA library was prepared from peripheral blood monocyte-derived macrophages as described in Tjoelker et al., *Nature*, 374:549-552 (1995). Clones from the library were randomly chosen and plasmid DNA was purified from individual clones. The sequence of approximately 300 to 500 bases from the end of DNA from each clone was determined on an automated sequencer (Model 373, Applied Biosystems, Foster City, CA) using primer JHSP6, which hybridizes to the plasmid vector pRc/CMV (Invitrogen, San Diego, CA) adjacent to the cDNA cloning site:

JHSP6: 5'-GACACTATAGAATAGGGC-3' (SEQ ID NO: 5)

The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences, including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala et al., *supra*), oviductal glycoprotein from sheep, cow, and humans (DeSouza et al., *supra*), and chitinases from parasite (*Oncocerca*, Genbank accession no. U14639), wasp (*Chelonus*, Genbank accession no.

U10422), plant (*Nicotiana*, Genbank accession no. X77111), and bacteria (*Serratia*, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a human chitinase homolog.

The DNA sequence of clone pMO-218 (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the human chitinase cDNA (nucleotides 2 to 1402 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the amino-terminal sequence of purified human chitotriosidase reported in Renkema *et al.*, *supra*. Renkema *et al.* also described a twenty-one amino acid sequence from a tryptic fragment of human chitotriosidase which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot *et al.*, *supra*, report the cloning of a human chitotriosidase cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot *et al.* by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

To confirm that MO-218 indeed contained the entire coding region of the cDNA, a ³²P-labelled probe P-1 (TGGGATCATCAGCAGGACCATGAAACCTGCCCAGGCCACAGACCGCACCAT, SEQ ID NO: 6) was prepared that corresponded to the complement of nucleotides 2 through 52 of MO-218 (SEQ ID NO: 1). Probe P-1 was designed to hybridize with clones that are at least as long as MO-218 at the 5' end. The probe was hybridized with a portion (approximately 30,000 clones) of the human macrophage cDNA library described above, in 40% formamide and hybridization buffer (5 x SSPE, 10 x Denhardt's, 100 µg/ml denatured salmon sperm DNA, and 2% SDS) at 42°C overnight. The filters were washed and three clones that hybridized were chosen for

sequence /analysis. The longest clone was designated pMO-13B (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98078). The DNA sequence of pMO-13B is set forth in SEQ ID NO: 3 and the encoded amino acid sequence is set forth in SEQ ID NO: 4. This clone contains 25 additional nucleotides at the 5' end compared with MO-218; in addition, MO-13B (SEQ ID NO: 3) contains one nucleotide substitution at nucleotide 330 (corresponding to nucleotide 305 of MO-218, SEQ ID NO: 1) which changes the encoded amino acid at position 80 of the mature protein from a glycine (in SEQ ID NO: 2) to a serine (in SEQ ID NO: 4).

Example 2

Chitinase Gene Expression Pattern in Human Tissues

Northern blot analysis was performed to identify tissues in which the human chitinase is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto CA) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

Example 3

Production of Recombinant Human Chitinase in Bacterial Cells

The mature coding region of MO-218 was engineered for expression in *E. coli* as a C-terminal truncated analog. PCR was used to generate a DNA fragment for expression using a primer corresponding to nucleotides 65 to 88 of the MO-218 chitinase cDNA preceded by an initiating methionine codon and an XbaI restriction endonuclease site (5'-

TACATCTAGAATTATGGCAAACTGGTCTGCTACTTCACC-3', SEQ ID NO: 7), and a downstream primer encoding nucleotides 1163 to 1183 of MO-218 followed by a stop codon and a HindIII site (5'-

AGATCTAACCTTAGGTGCCTGAAGACAAGTATGG-3', SEQ ID NO: 8). The downstream primer contained an adenine at base 25, while the MO-218 sequence contains a guanine at the corresponding nucleotide position. Consequently, the resulting DNA fragment contains a thymine rather than a cytosine at the position corresponding to nucleotide 1172 of the MO-218 sequence, and the encoded chitinase fragment, set forth in SEQ ID NO: 15, is also an analog that contains a serine at mature amino acid position 370 instead of the proline encoded by MO-218. The resulting DNA fragment was digested with XbaI and HindIII and cloned into plasmid pAraBAD (which is also known by the designation pAraCB).

Plasmid pAraCB was prepared as follows. Plasmid pUC19 was modified to include an arabinose promoter and subsequently to include AKAP 79 encoding sequences. The arabinose promoter [Wilcox *et al.*, *Gene* 34:123-128 (1985); Wilcox, *et al.*, *Gene* 18:157-163 (1982)] and the *araC* gene were amplified by PCR from the arabinose operon BAD of *Salmonella typhimurium* as an *EcoRI/XbaI* fragment with the primers *araC*-2 (SEQ ID NO: 9) and *arab*-1 (SEQ ID NO: 10):

araC-2 TACAGAATTC*TTATTC*ACATCCGGCCCTG SEQ ID NO: 9

arab-1 TACATCTAGACTCCATCCAGAAAAACAGGTATGG SEQ ID NO: 10

Primer *araC*-2 encodes an *EcoRI* site (underlined) and a termination codon (italics) for the *araC* gene product. Primer *arab*-1 encodes a putative ribosome binding domain

(italics) and an *Xba*I restriction site (underlined). PCR with these primers produced a 1.2 kb fragment which was digested with *Eco*RI and *Xba*I and subcloned into pUC19 (New England Biolabs, Beverly, MA) previously digested with the same two enzymes. The resulting plasmid was designated araCB and contained a polylinker region (SEQ ID NO: 11) flanked at the 5' end with a *Xba*I restriction site (underlined) and at the 3' end with a *Hind*III site (italics).

araCB polylinker

SEQ ID NO: 11

TCTAGAGTCGACCTGCAGGCATGCAAGCTT

Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37°C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length chitinase contains ten cysteine residues. The inclusion bodies were separated from the *E. coli* culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of human chitinase. The remaining material corresponded to a contaminating *E. coli* protein, porin. This recombinant chitinase preparation from *E. coli* was useful for producing polyclonal and monoclonal antibodies (described below in Example 7).

When transformants containing the Ara-chitinase expression plasmid were grown at 25°C, inclusion bodies were not observed and expression of recombinant product was decreased from about ten percent of total cell protein to about one percent. However, this material produced at 25°C exhibited chitinase catalytic activity.

Example 4

Production of Recombinant Human Chitinase in Yeast Cells

Exemplary protocols for the recombinant expression of human chitinase

in yeast and for the purification of the resulting recombinant protein follow. The

5 coding region of human chitinase is engineered into vectors for expression in
Saccharomyces cerevisiae using either PCR or linker oligonucleotides designed to
encode a fusion polypeptide containing a secretion mediating leader to the coding
region for human chitinase corresponding to the amino terminus of the natural
molecule. Secretion signal peptides include, *e.g.*, SUC2 or equivalent leaders with a
10 functional signal peptidase cleavage site, or pre-pro-alpha factor or other complex
leader composed of a pre, or signal peptide, and a pro, or spacer region, exhibiting a
KEX2 cleavage site. The DNA encoding the signal sequence can be obtained by
oligonucleotide synthesis or by PCR. The DNA encoding the pre-pro-alpha factor
leader is obtained by PCR using primers containing nucleotides 1 through 20 of the
15 alpha mating factor gene and a primer complementary to nucleotides 255 through 235
of this gene [Kurjan and Herskowitz, *Cell*, 30:933-943 (1982)]. The pre-pro-alpha
leader coding sequence and human chitinase coding sequence fragments are ligated
into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such
that the promoter directs the expression of a fusion protein. As taught by Rose and
20 Broach, [*Meth. Enz.*, 185:234-279, D. Goeddel, ed., Academic Press, Inc., San
Diego, CA (1990)], the vector further includes an ADH2 transcription terminator
downstream of the cloning site, the yeast "2-micron" replication origin, a selectable
marker, for example TRP1, CUP1 or LEU2 (or LEU2-d) or other equivalent gene,
the yeast REP1 and REP2 genes, the *E. coli* beta lactamase gene, and an *E. coli*

08663618-082896

origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

Alternatively, other fusion points within the chitinase coding region may be chosen. Truncates of the coding region may be used to increase homogeneity of the product, increase the specific activity or alter the substrate specificity.

The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, *e.g.* lithium acetate treatment [Stearns *et al.*, *Meth. Enz.*, *supra*, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price *et al.*, *Gene*, 55:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature human chitinase peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease [Bitter *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:5330-5334 (1984)].

Chitinase contains in its mature amino acid sequence two dibasic sequences at positions 107-108 (Lys-Arg) and 209-210 (Arg-Lys) that may be proteolytically clipped by the KEX2 protease during secretion. To stabilize and/or increase the level of product secreted from cells, these sequences could be mutated to eliminate the potential sites for proteolysis as shown by Gillis *et al.* [Behring Inst. Mitt., No. 83:1-7 (1988)] or by expressing chitinase without dibasic modifications in a host that is deficient in KEX2. Such hosts can be obtained either by screening for non-KEX2 protease containing mutants, or by manipulation of the genomic KEX2

08663418-082896

locus by gene replacement/gene disruption techniques [Orr-Weaver *et al.*, *Proc. Natl. Acad. Sci, USA*, 78:6354-6358 (1981)].

Recombinant chitinase may be secreted from *Saccharomyces cerevisiae* using similar vectors containing alternative promoters PRB1, GAL4, TPI, or other suitably strong promoters bearing fragments or by fusion to a variety of leader sequences [Sleep *et al.*, *Bio/Technol.*, 8:42-46 (1990)].

Other non-*Saccharomyces cerevisiae* suitable expression hosts include *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Pichia pastoris* and members of the *Hansenula* or *Aspergillus* geni. Analogous recombinant expression systems for these fungi include the organism and their appropriate autonomously replicating vector [e.g. Falcone *et al.*, *Plasmid*, 15:248-252 (1988)] or multiply integrated expression cassettes. These systems also rely on signal sequences or leaders of the types described above to mediate secretion into the medium.

The secreted recombinant human chitinase is purified from the yeast growth medium by, e.g., the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

Alternatively, the mature form of the recombinant chitinase product may be expressed in the cytoplasm of the *Saccharomyces cerevisiae* cells or analogous host, and purified from the lysed host cells. The protein may be refolded during the act of purification to obtain appropriate levels of specific activity.

Example 5

Production of Recombinant Human Chitinase in Mammalian cells

The MO-218 clone and the MO-13B clone, both of which contain full length human chitinase cDNA 3' to the CMV promoter of pRc/CMV, were isolated.

5 A third plasmid, which corresponded to the same C-terminal fragment expressed in bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1

(CGCAAGCTTGAGAGCTCCGTTCCGCCACATGGTGCGGTCTGTGGCCTGGG, SEQ ID NO: 12), which contains a Hind III site and nucleotides 2 through 23 of the

10 MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO:

13), which contains nucleotides 1164 through 1183 of MO-218, a stop codon and an XbaI site. The amplified DNA was purified by electrophoresis, digested with XbaI and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, CA)

15 previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, CA) and encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

All three plasmids were transiently transfected into COS cells by the DEAE transfection method [see, *e.g.*, Sambrook *et al.*, *Molecular Cloning: a*
20 *Laboratory Manual*, 2d ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).]. After three days at 37°C, media from cells was assayed for chitinase activity *in vitro* as described below in Example 8. Each culture produced significant chitinase activity (600-800 mU/ml/min), and similar amounts were observed for each construct.

Recombinant human chitinase was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95% of the chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85% pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree 10K, Millipore Corp., Bedford, MA). This preparation was then tested for enzymatic and anti-fungal activity *in vitro* as described in Examples 8 and 9 below. The recombinant preparation had a chitotriosidase activity of 90 nm/min per mg protein.

Example 6

Production of Human Chitinase Analogs and Fragments

Recombinant techniques such as those described in the preceding examples may be used to prepare human chitinase polypeptide analogs or fragments.

5 More particularly, polynucleotides encoding human chitinase are modified to encode polypeptide analogs of interest using well-known techniques, *e.g.*, site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions may also be prepared by, *e.g.*, deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook *et al.*, *supra*, Chapter 15. The modified
10 polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

Residues critical for human chitinase activity are identified, *e.g.*, by
homology to other chitinases and by substituting alanines for the native human
chitinase amino acid residues. Cysteines are often critical for the functional integrity
15 of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To determine whether any of the cysteines in human chitinase are critical for enzymatic activity, each cysteine is mutated individually to a serine.

A 39 kDa C-terminally truncated fragment of the mature human
chitinase protein was prepared as described above in Examples 3 and 5 by
20 introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant human chitinase. This result indicates that the missing

08663618.082896

seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

Further C-terminal deletions may be prepared, *e.g.*, by digesting the 3' end of the truncated human chitinase coding sequence described in Example 3 with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the 5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

Alternatively, human chitinase polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [See, *e.g.*, synthesis of IL-8 in Clark-Lewis *et al.*, *J. Biol Chem.*, 266:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis *et al.*, *Science*, 231:134-139 (1986); and synthesis by ligation in Dawson *et al.*, *Science*, 266:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the human chitinase polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 8 to 14 below.

Example 7

Preparation of Monoclonal Antibodies to Human Chitinase

The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to human chitinase.

- 5 In the first protocol, a mouse is injected periodically with recombinant human chitinase (*e.g.*, 10-20 μ g emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a final pre-fusion boost of human chitinase in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single
- 10 cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and is
- 15 washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11 % fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is
- 20 washed twice as described in the foregoing paragraph.

One $\times 10^8$ spleen cells are combined with 2.0×10^7 NS-1 cells and centrifuged, and the supernatant is aspirated. The cell pellet is dislodged by tapping the tube, and 1 ml of 37°C PEG 1500 (50% in 75mM Hepes, pH 8.0) (Boehringer Mannheim) is added with stirring over the course of 1 minute, followed by the

addition of 7 ml of serum-free RPMI over 7 minutes. An additional 8 ml RPMI is added and the cells are centrifuged at 200 g for 10 minutes. After discarding the supernatant, the pellet is resuspended in 200 ml RPMI containing 15% FBS, 100 μ M sodium hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5×10^6 splenocytes/ml and plated into 10 Corning flat-bottom 96-well tissue culture plates (Corning, Corning New York).

On days 2, 4, and 6, after the fusion, 100 μ l of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by ELISA, testing for the presence of mouse IgG binding to human chitinase as follows. Immulon 4 plates (Dynatech, Cambridge, MA) are coated for 2 hours at 37°C with 100 ng/well of human chitinase diluted in 25mM Tris, pH 7.5. The coating solution is aspirated and 200 μ l/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37°C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 μ l culture supernatant is added. After incubation at 37°C for 30 minutes, and washing as above, 50 μ l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 μ L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μ l/ml 30% H₂O₂ in 100 mM Citrate, pH 4.5, are added. The color reaction is stopped after 5 minutes with the addition of 50 μ l of 15% H₂SO₄. A₄₉₀ is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal

antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, IN).

Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, *Methods Enzymol.*, 121:33-41 (1986). The spleen of the animal is exposed and injected with recombinant human chitinase (*e.g.*, 10-20 μ g in PBS at a concentration of about 0.02% to 0.04%, with or without an aluminum adjuvant) obtained as described in any of Examples 3 through 6, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640 supplemented with 3% fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450 g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37°C) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 20% FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37°C in a humid atmosphere with 5% CO₂-95% air for 8 to 10 days. Supernatants are aspirated and

the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

Example 8

Catalytic Activity of Recombinant Chitinase

Chitotriosidase (chitinase) activity was measured using the fluorogenic substrate 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose (4 MU-chitotrioside, Sigma Chemical, St. Louis, MO) in McIlvain buffer (Hollak *et al.*, *supra*). Ten μ l samples of the recombinant product were combined with 10 μ l bovine serum albumin (10 mg/ml), 15 μ l fluorogenic substrate (2.71 mM), and 65 μ l buffer (0.1M citric acid, 0.2M sodium phosphate, pH 5.2) in a total volume of 100 μ l. Reactions were incubated at 37°C for 15 minutes, then the reaction was stopped with the addition of 2 ml of 0.3M glycine/NaOH buffer (pH 10.6). The fluorescent cleavage product, 4-methylumbelliferone, was monitored with a fluorimeter (SLM-AMINCO Instruments, Inc., Rochester, NY) at 450 nm. To obtain a standard curve, several substrate concentrations were combined with excess bacterial chitinase to ensure that substrate was completely cleaved. The known quantity of 4-MU was then correlated to the fluorescence signal from the fluorimeter and linear regression was used to determine a standard curve. The signal produced with diluted purified recombinant chitinase in the assay was then used to interpolate the nm quantity of substrate cleaved by the enzyme during the reaction time. This number was then divided by the concentration of protein to obtain the nm/min per mg protein (determined by A_{280} and calculated molar extinction coefficient).

The chitotriosidase activity of the recombinant human chitinase from Example 5 was determined to be 90 nm/min per mg protein.

Example 9

Anti-fungal Activity of Recombinant Chitinase *In Vitro*

5 In a preliminary experiment, recombinant human chitinase was tested for inhibition of fungal growth *in vitro*. The two fungi *Candida albicans* and *Aspergillus fumigatis* are serious pathogens for immunocompromised patients. Both *Candida* and *Aspergillus* were grown in RPMI growth media at 37°C to approximately 10,000-50,000 colony forming units (CFU) per ml. Recombinant human chitinase (prepared
10 as described in Example 5) was added to cultures at 0, 2.8, 11.25, or 45 µg/ml. After 24 hours, fungal growth was assessed by turbidity of cultures. Under these non-physiological conditions in this assay, all cultures appeared to grow at comparable rates, independent of chitinase concentration. The concentration of fungi tested, however, is much higher than the fungal burden seen during fungal infection *in vivo*.
15 Different results may be obtained under different conditions, *e.g.*, with a lower fungal burden, or when human chitinase is tested in combination with other anti-fungal agents. Chitinase is also expected to be more effective *in vivo* under physiological conditions.

08663618.082896

Example 10

Anti-fungal Activity of Recombinant Chitinase *In Vivo* in Mice

Several animal models have been developed for testing efficacy of anti-fungal compounds [see Louie et al., *Infect. Immun.*, 62: 2761-2772, 1994; Kinsman et al., *Antimicrobial Agents and Chemotherapy*, 37: 1243-1246, 1993; Nakajima et al., *Antimicrobial Agents and Chemotherapy* 39: 1517-1521, 1995; Tonetti et al., *Eur. J. Immunol.*, 25:1559-1565 (1995)]. Briefly, the animal host is infected with the fungi, varying doses of chitinase are administered to the animals, and their survival is measured over time. The experiments are performed using chitinase as the sole therapeutic agent, or with a combination of conventional anti-fungal agents such as Amphotericin B and fluconazole to determine if the chitinase improves the efficacy of such compounds. Specifically, acute systemic candidiasis is achieved in mice by intraperitoneal or intravenous challenge of 10×10^6 CFU *Candida albicans*. The therapeutic agents are administered before or at 1 to 5 hours after challenge, and the number of survivors is determined after five days. In addition, the mice can be sacrificed and fungal load can be determined in specific organs such as kidney, lung, liver and spleen. Alternatively, the mice are challenged with lower doses of *Candida* (1×10^6 CFU), in which case survival can be measured at more distant time points, e.g., 45 days. Effective anti-fungal agents enhance the long term survival of animals and reduce fungal load in blood and organs.

Example 11

Activity of Chitinase *In Vivo* in a Rabbit Model of Invasive Aspergillosis

08663618-082896

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in an immunosuppressed rabbit model of invasive aspergillosis which has been used for over ten years to evaluate a variety of anti-fungal therapies. See, *e.g.*, Andriole et al., *Clin. Infect. Dis.*, 14(Suppl. 1):S134-S138 (1992). The study is conducted generally according to Patterson et al., *Antimicrob. Agents Chemother.*, 37:2307-2310 (1993) or George et al., *J. Infect. Dis.*, 168:692-698 (1993). Briefly, on day one the rabbits are given cyclophosphamide (200 mg) intravenously to render them leukopenic, followed by triamcinolone acetonide (10 mg) subcutaneously each day for the duration of the experiment. On day two, 24 hours after immunosuppression, the animals are challenged intravenously with about 10^6 (lethal challenge) or about 10^5 (sublethal challenge) *A. fumigatus* conidia. Anti-fungal therapy (chitinase alone, or in combination with other conventional anti-fungal agents, *e.g.*, amphotericin B, fluconazole, or 5-fluorocytosine) is initiated at 24 hours after challenge or 48 hours before challenge (for prophylaxis) and is continued for 5 to 6 days or until death. Exemplary doses of conventional anti-fungal agents are 1.5 or 0.5 mg/kg/day intravenous amphotericin B, 60 or 120 mg/kg/day oral fluconazole and 100 mg/kg/day oral 5-fluorocytosine. Control rabbits are not treated with any anti-fungal agent.

At autopsy or death, semiquantitative fungal cultures and histopathologic examination are conducted on the liver, spleen, kidneys, lungs and brain. Cultures of the heart, urine and blood may also be performed. Blood samples

are obtained at intervals and assayed for white blood cell counts and circulating *Aspergillus* carbohydrate antigen using an ELISA assay. The effect of treatment with the test drug is evaluated on three endpoints: reduction in mortality rate, reduction in number of *Aspergillus* organisms cultured from target organs (fungal burden), and reduction in level of circulating *Aspergillus* antigen. Effective anti-fungal agents reduce mortality and/or fungal load.

Alternatively, pulmonary aspergillosis may be evaluated in this model generally according to Chilvers et al., *Mycopathologia*, 108:163-71 (1989), in which the immunosuppressed rabbits are challenged with intratracheal instillation of *Aspergillus fumigatus* conidia, followed by bronchoalveolar lavage on days 1, 2, 4, 7 and 10 following challenge; fungal culture, chitin assay, white cell counts and histopathology are performed on the lavage fluids to determine infective load within the lung. Effective fungal agents reduce the infective load or inflammation within the lung.

Example 12

Activity of Chitinase *In Vivo* in a Rabbit Model of Disseminated Candidiasis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of disseminated candidiasis generally according to Rouse et al., *Antimicrob. Agents Chemother.*, 36:56-58 (1992). New Zealand white rabbits are infected systemically with about 3×10^6 *Candida albicans* blastospores. Anti-fungal therapy is initiated 48 hours after challenge with *Candida* (or before challenge for prophylaxis) and is continued for,

5 e.g., four days. Surviving animals are sacrificed, and fungal cultures are performed on the aortic valve with attached vegetation, lung, kidney and spleen. Fungal cultures and histopathological examination may also be performed on these and other organs, such as liver, brain, and heart. Urine and blood cultures may also be done. The effect of the anti-fungal therapy on mortality and circulating or tissue fungal burden is determined.

960200 0105980
10 Bayer et al., *Antimicrob. Agents Chemother.*, 19:179-184 (1981), in which rabbits are inoculated intraperitoneally with about 5×10^8 CFU *Candida albicans*. A saline peritoneal aspirate is obtained and cultured from each animal four days after intraperitoneal inoculation, and animals with a positive fungal culture aspirate are randomly assigned to control or treatment groups. Anti-fungal treatment is begun seven days after challenge. The eyes of all rabbits are evaluated using indirect ophthalmoscopy, as disseminated candidiasis may result in *Candida* endophthalmitis. Animals are sacrificed at 7, 11 and 14 days after initiation of
15 therapy and their abdomens inspected for evidence of peritonitis and intraabdominal abscess formation. Eyes are examined for macroscopic lesions. Tissue samples from peritoneal abscesses, all other visible abscesses, kidneys, livers, spleens and ocular structures are weighed, homogenized in brain heart infusion broth, serially diluted and cultured to determine the CFU per gram of tissue. Renal and peritoneal abscesses are
20 also fixed in 10% neutral formaldehyde and examined for histopathology. Sections are stained with periodic acid-Schiff reagent to determine the fungal burden and fungal morphology. Effect of the test drugs on improving survival and reducing fungal burden is evaluated.

Example 13

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endophthalmitis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endophthalmitis, generally according to Park et al., *Antimicrob. Agents Chemother.*, 39:958-963 (1995). Briefly, New Zealand albino rabbits, 2 to 2.5 kg, are infected with an intravitreal inoculation of about 1,000 CFU of *Candida albicans*. Endophthalmitis is confirmed 5 days after inoculation by indirect ophthalmoscopy, and is defined as moderate to severe vitreous haze with partial or complete obscuration of greater than 50% of the retinal and choroidal vasculature. The vitreous turbidity is graded on a scale, and the fundus appearance may be graded and documented by fundus photography. The rabbits are then randomized to the following treatment conditions: chitinase alone for 2 to 4 weeks, a combination of chitinase and another conventional anti-fungal agent (*e.g.*, amphotericin B, fluconazole or 5-fluorocytosine) for 2 to 4 weeks, or no treatment (control). Exemplary doses of conventional anti-fungal agents are 80 mg/kg/day of oral fluconazole and 100 mg/kg every 12 hours of oral 5-fluorocytosine.

The treatment effect is assessed at 2 and 4 weeks after therapy by indirect ophthalmoscopy, quantitative fungal culture, and histopathology. For quantitative fungal culture, the eyes are dissected and weighed, and a weighed fraction of each sample is homogenized and cultured on brucella agar-5% horse blood plates for 48 hours at 35°C in 5 to 10% CO₂. The homogenized sample may also be diluted 10- or 100-fold with sterile saline before plating. The colonies are counted

and the total CFU in the eye calculated on the basis of the growth yielded from the measured fractions of sample. Treatment effect is assessed in terms of a reduction in the total intraocular fungal burden. For histopathology, representative eyes are removed, fixed in formalin, embedded in plastic, and sliced into 5 μ m sections. The sections are stained with hematoxylin-eosin or Gomori's methenamine silver stain and examined by light microscopy for inflammation, fibrous organization and fungal elements. The effect of the anti-fungal agents on reducing mortality, reducing fungal load, or reducing the inflammation associated with fungal infection, is evaluated.

Alternatively, a rabbit model of *Aspergillus* endophthalmitis may be used generally according to Jain et al., *Doc. Ophthalmol.*, 69:227-235 (1988). Briefly, New Zealand white rabbits are inoculated in one eye with about forty spores of *Aspergillus fumigatus*. Their contralateral (control) eyes receive a similar but sterile inoculum. After treatment with the test drug (chitinase alone, or chitinase in combination with another agent), the rabbits' eyes may be evaluated for clinical appearance, electroretinogram waveforms, indirect ophthalmoscopy, quantitative fungal culture, and histopathology. Clinically evident endophthalmitis typically develops within three to seven days after inoculation.

Example 14

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endocarditis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endocarditis generally according to Witt and Bayer, *Antimicrob. Agents Chemother.*, 35:2481-2485

(1991). See also Longman et al., *Rev. Infect. Dis.*, 12(Suppl. 3):S294-298 (1990).

08663618-082896
Sterile thrombotic endocarditis is produced in New Zealand white rabbits by
transaortic valvular placement of a sterile polyethylene catheter (internal diameter,
0.86 mm), which remained in place for the duration of the study. Infective
5 endocarditis is then established 48 hours after catheterization by intravenous injection
of about 2×10^7 *C. albicans* blastospores. Alternatively, *C. parapsilosis* may be
used. Anti-fungal therapy (chitinase or chitinase in combination with another
conventional anti-fungal agent) is initiated either 24 hours before or 24 to 60 hours
after fungal challenge. Therapy is continued daily for 9 or 12 days. Exemplary
10 doses of conventional anti-fungal agents are 1 mg/kg/day intravenous amphotericin B,
50 mg/kg/day or 100 mg/kg/day intravenous or intraperitoneal fluconazole. Control
rabbits are given no anti-fungal agent. At sacrifice, hearts are removed and the
position of the indwelling catheter verified. Cardiac vegetations from each animal are
removed, pooled, weighed and homogenized in 1 ml of sterile saline. The
15 homogenate is serially diluted and quantitatively cultured on yeast potassium dextrose
agar at 35°C for 48 hours. Culture-negative vegetations are considered to contain
less than $2 \log_{10}$ CFU/gram on the basis of average vegetation weight.

Numerous modifications and variations of the above-described invention
are expected to occur to those of skill in the art. Accordingly, only such limitations
20 as appear in the appended claims should be placed thereon.